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Genome-wide transcriptomic effects of phytoestrogens in breast cancer cells

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Meinen Eltern.

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1. Summary

The nutritional intake of phytoestrogens seems to reduce the risk of breast cancer or other neoplastic diseases. However, these epidemiologic findings are controversial because low phytoestrogen doses, achievable through soy-rich diets, stimulate the proliferation of estrogen-sensitive tumor cells. The question of whether such phytochemicals prevent cancer, or pose additional health hazards, led us to monitor global gene expression changes induced by phytoestrogens (daidzein, coumestrol, enterolactone, resveratrol) or a typical soy product (soymilk), from which the phytochemicals were extracted by reverse/normal phase chromatography. In each case, phytoestrogens were used to treat human cells representing a common model system for mammary tumorigenesis. Analysis of messenger RNA on high-density microarrays revealed that soy phytoestrogens induce a genomic fingerprint that is indistinguishable from the transcriptional effects of the physiologic hormone 17β-estradiol. Highly congruent responses were also observed by comparing the physiologic estradiol with phytoestrogen standards. More diverging transcriptional profiles were generated when an inducible promoter was used to reconstitute the expression of estrogen receptor β. We conclude that phytoestrogens mitigate estrogenic signaling in the presence of both estrogen receptor subtypes but, in late-stage cancer cells lacking estrogen receptor β, these phytochemicals may contribute to a tumor-promoting transcriptional signature.

2. Introduction

2.1 Estrogen receptors

2.1.1 Structure

There are two principal intracellular receptors that mediate the biological effects of estrogens: estrogen receptor α (ERα) and estrogen receptor β (ERβ). ERs belong to the steroid nuclear receptor superfamily, one of the largest protein families with more than 70 currently recognised members (Mangelsdorf *et al*., 1995). Nuclear receptors are ligand-activated transcription factors that regulate the expression of target genes involved in metabolism, cell proliferation, differentiation, growth, reproduction and development (Mangelsdorf *et al*., 1995; Enmark & Gustafsson, 1999; Penot *et al*., 2005; Moutsatsou, 2007).
ERα and ERβ are both composed of five independent but interacting functional domains (Fig. 1): the N-terminal domain (A/B), the DNA-binding domain (C), the hinge region (D), the ligand-binding domain (E) and the C-terminal domain (F).

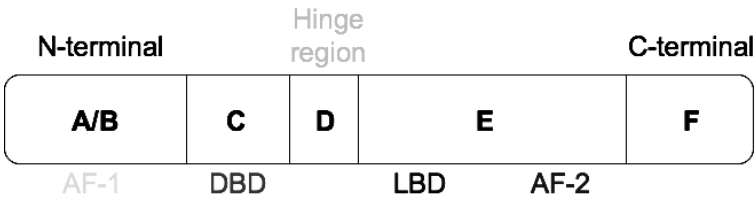


Figure 1: *Schematic representation of human estrogen receptors*

The N-terminal domains exhibit strong distinctions between ERα and ERβ (they share less than 20% amino acid identity) and contain the ligand-independent transcription activation function (AF-1), which is responsible for recruiting transcriptional co-activator proteins and exerts promoter- and cell specific activity. The ER subtypes α and β differ in the activity of AF-1: ERαAF-1 is very active in the stimulation of target gene expression from a variety of estrogen responsive reporter constructs, whereas the activity of ERβAF-1, under the same conditions, is generally reduced (Webb *et al.*, 1999; Kuiper *et al.*, 1996; Nilsson, 2001; Howell A, 2006).

In contrast, the DBD (DNA-binding domain) is a highly conserved structure where ERα and ERβ share a high degree of sequence identity (Matthews, 2003). The DBD consists of two zinc fingers that participate in receptor dimerisation (by forming hydrogen and ionic bonds with the zinc fingers of another ER) and mediate the binding to specific DNA sequences, termed HRE (hormone-responsive elements, i. e. ERE, for estrogen-responsive elements), within the promoter region of target genes.

ERα and ERβ share 56% sequence identity (Koehler *et al.*, 2005) in the ligand-binding domain (LBD), which lies in the C-terminal half of the receptor protein and includes the ligand-dependent AF-2 (activation function 2). AF-2 performs a direct ligand interaction and mediates receptor dimerisation, nuclear translocation and the recruitment of co-activator proteins (Muellller *et al.*, 2004; Barkhem *et al.*, 2002) leading to transactivation of target genes (Jacobs, 2002; Nilsson, 2001). The highest performance, i. e. full transcriptional activity, of the ligand-bound ER is achieved when AF-1 and AF-2 cooperate in a synergistic manner (Wärnmark & Gustaffson, 2003). Crystallographic studies with ERα and ERβ revealed that the AF-2 interaction surface is altered in its conformation

by the binding of specific ligands. Also, different ligands induce different receptor conformations that allow discrimination between ER agonists and antagonists. Only the agonistic interaction leads to the recruitment of co-activators (Nilsson, 2001).

The ligand specificity of ERs is determined by the surface chemistry of the binding cavity: amino acid residues lining the binding site interact with functional groups of the ligand molecule. Although ERα and ERβ vary to a great extend in the LBD, the amino acids of the binding cavity are much more highly conserved (Ekena *et al.*, 1998; Koehler *et al.*, 2005). Furthermore, the three-dimensional structures of the two ER subtypes are very similar despite the fact that the overall ligand-binding cavity of ERβ is about 20% smaller than that of ERα. These subtle differences are thought to be responsible for the selective affinity and pharmacologic activity of distinct ligands.

Between the DBD and LBD lies the flexible hinge region of the ER which plays a role in the sterical orientation of the nuclear receptor. It appears to be important for the nuclear translocation and has been reported to contain a nuclear localization signal (Picard *et al.*,1990). The C-terminal F-domain of the receptor protein displays less than 20% amino acid identity between the two ER subtypes and the functions of this domain remain undefined. Koide *et al.* (2007) postulate that the F-domain of ERα modulates the response to ligands by regulating the interaction with transcriptional co-activators.

Full-length ERα consists of 595 amino acids, whereas full-length ERβ contains only 530 amino acids (Penot *et al.*, 2005). For both receptor subtypes, however, different isoforms that vary in their molecular activity and biological function (Omoto *et al.*, 2003) have been reported. Some of these isoforms have extended amino-termini, others have truncations and/or

insertions at the carboxy-terminus and in the LBD region (Leygue *et al.*, 1996; Ogawa *et al.*, 1998; Moore *et al.*, 1998; Nilsson *et al.*, 2001; Tong *et al.*, 2002; Matthews & Gustafsson, 2003).

2.1.2 Mechanism of action

In the absence of ligands, the inactive ERs are sequestered in a multiprotein complex containing various molecular chaperones, including heat shock proteins (Pratt, 1997), in the nuclear compartment. The interaction with ligands induces the removal of chaperones (Hall, 2001; McDonnel, 2002), thus inducing conformational changes of the ER proteins that lead to dimerisation (either forming homo- or heterodimers, ERα-ERα, ERβ-ERβ or ERβ-ERα). These active ER dimers bind to estrogen-responsive elements (ERE) on the DNA and allow the recruitment of co-regulatory proteins and other transcription factors to induce or repress the transcription of genes (Pace *et al.*, 1997; Matthews & Gustafsson, 2003; Koehler *et al.*, 2005; Suzuki *et al.*, 2007). Each ERE acts as a cis-acting enhancer or transcriptional repressor located within the regulatory region of target genes (Hall, 2002, Jacobs, 2002; Nilsson, 2001). Binding of an agonist triggers AF-2 activity, leading to the recruitment of co-activators, whereas binding of an antagonist does not (Nilsson *et al.*, 2001; Matthews & Gustafsson, 2003).
There are also hints for signalling pathways that deviate from this classical model (see Figure 2). In fact, about one third of the genes known to be regulated by ERs in human cells do not contain ERE sequences, indicating additional mechanisms of gene regulation (Björnström & Sjöberg, 2005). One non-classical mode of action by which ERs can exert their influence

on gene transcription is a process referred to as transcription factor cross talk/ERE-independent genomic actions. Following this model, ERs interact in the presence of E2 with general transcripton factors such as AP-1 (activating protein 1) or Sp-1 (stimulating protein 1), thus forming multiprotein complexes that also regulate transcription of genes that do not contain EREs in their promoter region (Salvatori *et al.*, 2003). For example co-factors that function as bridging elements between ERα and AP-1 response elements (Barkhem *et al.*, 2002) can trigger or enhance transcription of genes that do not have an ERE. AP-1 transcription factors are considered immediate early-response genes involved in a wide range of regulatory processes linked to cellular proliferation and differentiation. AP-1 is the gene product of the Jun and Fos oncogenes and is composed of either homo- or heterodimers of the Jun and Fos family members. Examples of factors induced by ERα/AP-1 cross-talks include IGF-I, collagenase and cyclin D1. Conversely, the choline acetyltransferase gene is repressed by the ER/AP-1 complex. The interaction of ERs with Sp-1 results in the induction of LDL-R (low-density lipoprotein receptor), c-fos and cyclin D1 (Nilsson, 2001; Barkhem *et al.*, 2002; Björnström & Sjöberg, 2005).

There is also a ligand-independent action of ERs, which is modulated by cell cycle regulators, extracellular signals (cytokines, neurotransmitters, peptide growth factors) and protein kinases, resulting in phosphorylation of ERs in the absence of estradiol (Nilsson *et al.*, 2001; Hall *et al.*, 2001; Björnström & Sjöberg, 2005; Butt *et al.*, 2005).

Non-genomic actions are associated with a non-nuclear fraction of ERs, likely to be located in the cytosol or bound to the cell membrane, that mediates a rapid, transcription-independent response to estrogens leading to altered functions of cellular proteins. Non-genomic effects are for example

observed in endothelial cells, where estradiol leads to the release of nitric oxide (NO), or in neurons, where estradiol induces neuroprotective effects via MAPK-activation (Moggs & Orphanides, 2001; Nilsson, 2001; Björnström & Sjöberg, 2005).

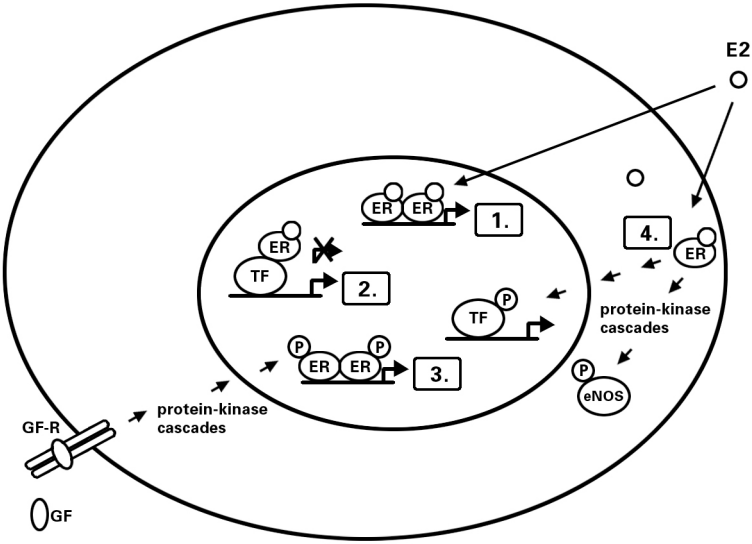


Figure 2:
Schematic illustration of different ER signalling mechanisms

1. Classical mechanism of ER action: ligand-activated ERs bind directly to EREs in target gene promoters. 2. ERE-independent genomic actions: ER complexes are tethered through protein-protein interactions to a transcription factor complex (TF) that makes contacts with the target gene promoter. 3. Ligand-independent genomic actions: growth factors (GF) activate protein kinase cascades, leading to

*phosphorylation (P) and activation of nuclear ERs. 4. Non-genomic actions: Membrane-bound ERs activate protein-kinase cascades, leading to altered functions of proteins in the cytoplasm, e.g. activation of eNOS (*endothelial nitric oxide synthase)*, or to regulation of gene expression through phosphorylation (adapted from Björnström & Sjöberg, 2005).*

2.1.3 Ligands

Endogenous estrogens (primarily 17β-estradiol) are the natural and best fitting ligands of the ERs. Nevertheless, other similar molecules, like phytoestrogens, are potential ligands for the ERs as long as their structure resembles that of 17β-estradiol (Nilsson *et al.*, 2001). Common structural elements of the phytoestrogens and 17β-estradiol include a phenolic ring with a pair of hydroxyl groups, which are a precondition for binding to ERs. DES (diethylstilbestrol), which is lacking the steroid structure, is still able to bind to ERs in light of its two symmetric phenolic rings. DES (containing two phenolic rings) and resveratrol (containing one phenolic and one dihydroxylated phenolic ring) show high structural similarity, but differ significantly in their relative binding affinities for ERα and ERβ (Table 1).

The binding affinity of a ligand to a certain receptor type is unable to predict whether the effect is agonistic or antagonistic (Bowers *et al.,* 2000*;* Mueller *et al.*, 2004), i.e., to predict whether the ligand up- or down regulates the transcription of target genes. The effect exerted by a specific ligand can be assessed by comparing downstream transcriptional activities. Mueller *et al.* concluded that coumestrol, equol (a human metabolite of daidzein) and resveratrol are, like DES and 17β-

estradiol, full receptor agonists. Enterolactone, on the other hand, was classified as a partial agonist and partial antagonist because it reduces the transcriptional activity of DES bound to ERs. Similarly, resveratrol was identified as a mixed ER agonist/antagonist: at low doses (up to 10 μM) it increases the DES-induced ER activity but at high doses (100 μM) resveratrol inhibits the activity of ERs.

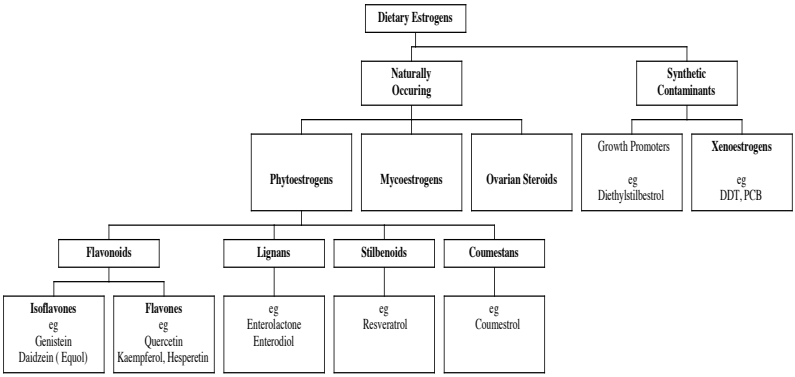


Figure 3: *Classification of dietary estrogens*

Phytoestrogens are polyphenolic non-steroidal plant compounds with estrogen-like biological activity, present in several edible plants. On the basis of their chemical structure, phytoestrogens may be divided into four subclasses: flavonoids (several subgroups: e.g. isoflavones, flavones), stilbenoids, coumestans, and lignans (Figure 3). The major dietary sources of isoflavones (e.g. genistein, daidzein, formononetin, biochanin A) are soybeans, legumes in general, chickpeas and peanuts. Flavones (e.g. chrysin, apigenin, naringenin, quercetin) are more widely distributed in the plant kingdom and

are present in citrus fruits, green tea, gingko, chocolate and wine. Coumestans (e.g. coumestrol, wedelolactone) are present for example in legumes, spinach, clover, brussel sprouts and also in alfalfa sprouts. Mammalian lignans (e.g. enterolactone and enterodiol) are not present in the human diets as such, but are ingested as precursors (plant lignans), which are converted to mammalian lignans by gut microflora. Plant lignans (e.g. pinoresinol, lariciresinol, secoisolariciresinol, matairesinol, sesamin) are present in fiber-rich foods such as flaxseed, sesame seed, pumpkin seed, broccoli and grain products. Stilbenoids, like resveratrol, can be found mainly in the skin of red grapes and unprocessed peanuts (Murkies, 1998, Nilsson *et al*., 2001; Moutsatsou, 2007).

	ERα IC_{50}	RBA (ERα)	ERβ IC_{50}	RBA (ERβ)
DES	4.6 ± 1.0 nM	100	4.6 ± 1.5 nM	100
E2	4.3 ± 1.1 nM	107	5.7 ± 0.7 nM	82
Coumestrol	38 ± 15 nM	12	6.0 ± 3.6 nM	77
Enterolactone	6.7 ± 4.3 μM	0.07	39 ± 22 μM	0.01
Equol (Daidzein)	1.5 ± 0.5 μM	0.3	0.2 ± 0.01 μM	3
Resveratrol	7.7 ± 2.3 μM	0.06	29 ± 20 μM	0.02

Table 1:
Relative binding affinities (RBA) of estradiol and phytoestrogens for human ERα and ERβ (Mueller *et al*., 2004)

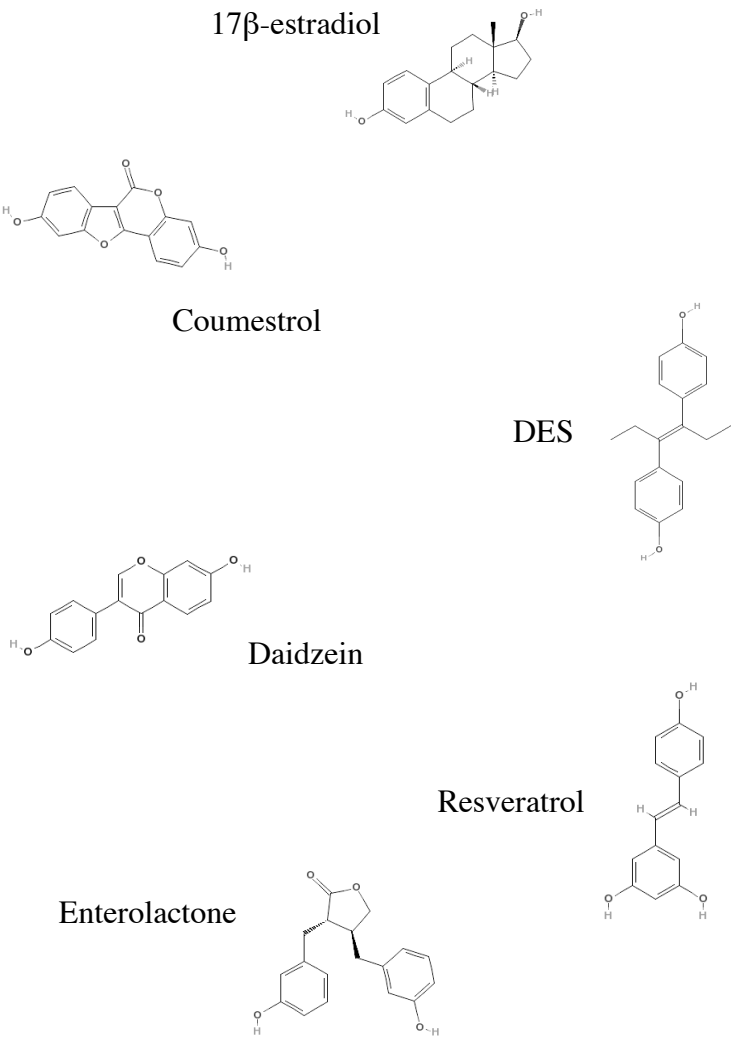


Figure 4:
Chemical structure of the phytoestrogens coumestrol, daidzein, enterolactone, resveratrol, the endogenous hormone 17β-estradiol and DES

2.1.4 Distribution of the estrogen receptors

An important aspect in understanding the estrogenic effects of phytoestrogens is the different tissue distribution and the level of expression of the two ERs. Although the ERs show high homology in their molecular structure, their tissue distribution is very variable (Kuiper *et al*., 1997; Gustafsson *et al*, 1999). While ERα is mainly expressed in the uterus, vagina, kidney and liver, ERβ is predominantly expressed in the cardiovascular system, urinary tract, lungs, thyroid, skin, gastro-intestinal tract and cartilage as well as bones. In humans, an almost concomitant expression of the two ERs can be found in the central nervous system, in the mammary tissue, ovaries and testes.

For a better understanding of the multiple biological functions of the respective estrogen receptors, homozygous ERα, ERβ and ERαβ knockout (KO) mice have been generated using gene-targeting techniques (Krege *et al*., 1998, Couse & Korach, 1999; Enmark & Gustafsson, 1999; Walker & Korach, 2004). All three KO models were viable. The ERαKO, ERβKO and ERαβKO offspring develop in their genetically determined sexes with no morphological peculiarities in their reproductive tracts and organs, which is proving that the phenotypical sex does not depend on ER status.

However effects resulting from the respective ER knockout(s) appear with onset of puberty. In ERαKO and ERαβKO, both sexes are infertile. The uteri of the ERαKO and ERαβKO are insensitive to estradiol and hypoplastic in all major uterine constituents (myometrium, endometrial stroma, epithelium), resulting in uterine weights that are approximately one half the expected size relative to the wild-type littermates (Walker & Korach, 2004). Their ovaries are anovulatory, secrete increased amounts of estradiol, testosterone and FSH (follicle-stimulating

hormone) and develop hemorrhagic cysts due to chronic elevated LH (luteinizing hormone). This dysregulation of hormone homeostasis is due to the loss of ERα function in the hypothalamus, which leads to a failure of the negative feedback action of estradiol on the hypothalamic-pituitary axis (Couse *et al.*, 2003; Walker & Korach, 2004). The testes of the male ERαKO and ERαβKO have normal morphology but after puberty they show progressively decreasing weight, the sperms have poor motility and the seminiferous tubules exhibit dilatation due to fluid retention.

In the ERβKO mice, the females show reduced fertility that manifests itself in infrequent pregnancies and reduced litters. The males show normal morphology and fertility when compared to wild-type controls. In accordance with these findings, there are no obvious morphological phenotypes in ERβKO males and only slight alterations of the ovaries, resulting in a reduced ovulation rate, can be found in the female ERβKO mice. The uterus is of physiological structure and hormone responsiveness, and allows for normal pregnancies. The mammary gland does also exhibit normal structure and supports normal lactation (Krege *et al.*, 1998). In the double knockout mice (ERαβKO), the granulosa cells of some follicles in the ovaries undergo differentiation into Sertoli-like cells and resemble the seminiferous tubules of the testis, suggesting the possibility of an adult sex reversal (Couse, 1999). The mammary glands of the ERαKO and ERαβKO mice are immature and remain rudimentary after puberty. As the elevated estrogen levels do not result in an increased progesterone receptor gene expression and the prolactine serum concentration remains very low compared to wild-type littermates, this effect is also attributable to disruption of the hypothalamic-pituitary axis (Walker & Korach, 2004). The mating behaviour of male and female

ERβKO mice is not altered when compared to wild-type controls. However, both sexes of the ERαKO and ERαβKO are disturbed in their mating behaviour (Couse *et al*., 2001).

2.2 Phytoestrogens as endocrine disruptors

Endocrine disruptors are exogenous chemicals that possess the ability to interfere with the endocrine system either by mimicking or antagonising the effects of endogenous hormones. Endocrine-disrupting chemicals are thought to cause a wide range of adverse health effects, including reproductive deficits, developmental disorders and cancer (Colborn *et al*., 1993; Cooper *et al*, 1997; McLachlan, 2001; Jobling, 2006; Caserta *et al*, 2007). The US Environmental Protection Agency defines an endocrine disruptor as an „exogenous agent that interferes with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintainance of homeostasis, reproduction, development and/or behaviour“ (Jacobs, 2001).
Endocrine disruptors comprise a variety of chemical classes including several pharmaceutical agents, synthetic hormones (DES), plant metabolites (phytoestrogens), fungal metabolites (mycoestrogens), pesticides, fungicides or herbicides and many industrial products and pollutants (Lathers, 2002). Endocrine-disrupting chemicals are almost ubiquitously dispersed in the environment. Some of these compounds are highly persistent and have the potential for bioaccumulation and global distribution by long-distance transport. Other endocrine disruptors are rapidly degraded in the environment or by the organisms that incorporate them (Cooper, 1997; Safe, 2004; Jacobs, 2001; McLachlan, 2001; Campi, 2007). There is growing awareness for possible risks of such substances among

consumers, authorities, environmental organisations and other stakeholders (Colborn *et al.*, 1993; Cassidy, 2003).

The present thesis is concerned with the effects of phytoestrogens in modulating the function of ERα and ERβ. Previous epidemiological studies have linked an increased risk of developing mammary or endometrial malignancies to prolonged estrogen exposure associated with early menarche, oral contraceptives, nulliparity, late first-time pregnancy, delayed menopause or an estrogen replacement therapy (McPherson *et al.*, 2000; Clemons & Gross, 2001; Nelson *et al.*, 2002; Parodi *et al.*, 2005). In contrast, the elevated dietary intake of soy isoflavones such as genistein and daidzein correlates with a lower incidence of breast and prostate cancer (Ingram *et al.*, 1997; Murkies *et al.*, 1998). In Asian countries, where soy provides a main food source, breast cancer incidence is two thirds lower compared to western countries. These epidemiologic findings led to the perception that phytoestrogen-rich foods and food supplements are generally healthy (Balk, 2002; Rice, 2006). Therefore, various soy products have become extremely popular, including soy oil, soy isoflavones as dietary supplements and as remedy for menopausal discomfort, soy flour, soy-based infant formula, tofu and soymilk. However, the true health effects of phytoestrogens from soy or other sources remain highly controversial. Some studies indicate that they exert beneficiary effects in preventing breast cancer, other reports suggest that the same compounds may be able to promote breast cancer or have other adverse effects on the endocrine system (Setchell *et al.*, 1998; Glazier *et al.*, 2001; Parodi *et al.*, 2005; Patisaul, 2005; Ju, 2006; Moutsatsou, 2007; Wuttke *et al.*, 2007; Sebastian *et al.*, 2007; Gallo *et al.*, 2007; Duffy *et al.*, 2007).

In the normal resting mammary gland estrogen receptors are expressed in only a small proportion of epithelial cells that are

largely non-dividing (Ali & Coombes, 2002). In contrast, enhanced expression of ERs is a critical event in the pathogenesis of a majority (~ 70%) of breast cancers and, accordingly, the growth of malignant mammary tumours is estrogen-dependent in most cases (Hayashi *et al.,* 1997; Gruvberger *et al.*, 2001; Rice & Whitehead, 2006). Like other ER agonists, phytoestrogens stimulate the proliferation of estrogen-sensitive tumor cells in various experimental systems (Hsie *et al.,* 1998; Allred *et al.,* 2001) and this growth-promoting activity has raised concerns that soy products, or other dietary components containing similar phytochemicals, may represent an additional health hazard for vulnerable risk groups (Messina *et al.,* 2006). More scepticism regarding the true benefits of phytoestrogens came from the observation that hyperplasia of the mammary epithelium is detectable in breast biopsies of pre- and postmenopausal women after a period of dietary soy supplementation (Petrakis *et al.,* 1996; Hargreaves *et al.,* 1999). Thus, establishing the consequences of soy intake in specific populations at high risk for breast cancer is an important public health issue (Messina *et al.,* 2006).

2.3 Aim of the study

The aim of this study was to assess the ERα/ERβ mediated effects on gene regulation of some representative phytoestrogens (enterolactone, resveratrol, daidzein, coumestrol) both as single compounds and as complex dietary mixtures in vitro. To achieve comparability of the results, we also determined the effects of the endogenous hormone 17β-estradiol. Genome-wide transcriptional profiles have been determined in an estrogen-dependent human cell line (MCF7) that often serves as a model system to study cellular processes

related to cancer progression in the breast. The distinction between ERβ– and ERα–mediated effects was achieved with an ERβ-inducible T47D cell line.

3. Materials

3.1 Cell culture

Cell culture media (DMEM, Ham's F12, MEM) were obtained from Invitrogen, Grand Island, USA. FBS was purchased from HyClone Laboratories, USA; the xenoestrogen-free plastics were from Corning Inc., Grand Island, USA.

PUCK's EDTA

0.40 g KCl
8 g NaCl
0.35 g $NaHCO_3$
1 g D-Glucose
0.255 g 0.02 % EDTA
ad 1000 ml aqua dest.
Filtered through a 0.2 μm filter and stored at 4°C.

3.2 Chemicals and equipment

Chemicals:

The antibiotics (gentamycin, tetracycline, streptomycin, penicillin) were from Invitrogen. Daidzein and resveratrol were purchased from Sigma-Aldrich, St Louis, USA; 17β-estradiol, coumestrol and enterolactone were purchased from Fluka (Buchs, Switzerland).

Technical equipment for transcriptomics:

Agilent 2100 Bioanalyzer: Agilent Technologies, Inc., Santa Clara, USA
Hybridization Oven 640: Affymetrix®, P/N 8000139 (220V)
Fluidics Station 400: Affymetrix®, P/N 00-0079
GeneChip Scanner 3000: Affymetrix®, P/N 00-00212

Reagents and solutions used for hybridization, washing and staining of the DNA microarrays:

10 mg/mL goat IgG stock

50 mg goat IgG are resuspended in 5 ml of 150 mM NaCl and stored at 4°C.

Wash buffer A (non-stringent wash buffer), 1000 ml:

(6x SSPE, 0.01% Tween-20)
300 ml of 20x SSPE
1 ml of 10% Tween-20
699 ml of water
Filtered through a 0.2 μm filter

Wash buffer B (stringent wash buffer), 1000 ml:

(100 mM MES, 0.1 M NaCl, 0.01% Tween-20)
83.3 ml of 12x MES stock buffer
5.2 ml of 5 M NaCl
1 ml of 10% Tween-20
910.5 ml of water
Filtered through a 0.2 μm filter, stored at 4°C and shielded from light

2x Stain buffer, 250 mL:

(100 mM MES, 1 M NaCl, 0.05% Tween-20)
41.7 ml of 12x MES stock buffer
92.5 ml of 5 M NaCl
2.5 ml of 10% Tween-20
113.3 ml of water

12x MES stock buffer, 1000 ml, pH 6.5 – 6.7

(1.22 M MES, 0.89 M NaCl)
64.61 g of MES hydrate
193.3 g of MES sodium salt
800 ml of water
The volume is adjusted to 1000 ml and the solution is filtered through a 0.2 μm filter.

2x Hybridization buffer, 50 ml

(Final concentration is 100 mM MES, 1 M NaCl, 20 mM EDTA, 0.01% Tween-20)
8.3 ml of 12x MES stock buffer
17.7 ml of 5 M NaCl
4 ml of 0.5 M EDTA
0.1 ml of 10% Tween-20
19.9 ml of water
Stored at 4°C and shielded from light

3.3 Kits

The following kits were purchased from Affymetrix, Santa Clara, USA:

- RNeasy Mini kit: QIAGEN®, P/N 79306
- IVT Labeling kit4
- One-Cycle cDNA Synthesis kit
  - Sample Cleanup Module
  - Poly–A RNA Control kit
  - Hybridization Control kit
  - Hybridization, Wash and Stain kit

3.4 Microarrays

The Affymetrix HG-U133 Plus 2.0 microarrays allow for the analysis of the expression level of approximately 40'000 human transcripts. The oligonucleotide probes, 25 residues in length, are bound to a solid support. Eleven pairs of oligonucleotide probes are used to measure the level of transcription of each sequence. To detect background fluorescence resulting from unspecific hybridisation, a paired mismatch probe for every perfect match probe is included on the array.

4. Methods

4.1 Cell culture

The **MCF7** cell line, subtype **BUS**, kindly provided by Dr. Anna Soto, Tufts University, Boston, USA, is derived from a human female breast adenocarcinoma. It is positive for the ERα and β, though ERα expression is highly predominant (Vladusic, 2000). It is an estrogen-dependent cell line with a doubling time of approximately 25 to 30 hours. Cell stocks were stored in liquid nitrogen.

Prior to seeding, cells were thawed at 37 °C, transferred into sterile tubes (Falcon's 50 ml), diluted with warm complete DMEM (Dulbecco's Modified Eagle's Medium, containing 1000 mg/l glucose) and supplemented with 10% fetal bovine serum (FBS, Invitrogen), 0.1 U/ml penicillin and 0.1 mg/ml streptomycin). The cells were collected by centrifugation (5 minutes, 1000 rpm, 4 °C), resuspended in complete warm medium and transferred into 75 cm^2 xenoestrogen-free cell culture flasks (Corning Inc., Grand Island, USA). The cell culture was carried out at 37°C in saturated humid atmosphere under 5% CO_2. The culture medium was changed every 12 – 20 hours.

After the cells grew to 85 – 95% confluence (every second to third day), they were split at a ratio of 1:3. For subculturing, the cells were washed with prewarmed sterile PUCK's EDTA, trypsinised (1:30 dilution with PUCK's EDTA), collected in 10 ml complete medium and centrifuged for 5 minutes at 1'000 rpm. The supernatant was removed, the cell pellet was resuspended in warm complete medium and transferred to the cell culture flasks.

Before each experiment, the cells were rinsed with 37°C warm

PUCK's EDTA and the complete DMEM medium was changed to phenol red-free assay medium supplemented with 5% charcoal/dextran-stripped FBS (DCC-FBS) containing the previously indicated antibiotics. The cells remained for 48 hours in this estrogen-deprived milieu, with a medium change after 24 hours. Finally, test compounds were dissolved in dimethyl sulfoxide (DMSO) and added to the estrogen-free assay medium. The final DMSO concentration was adjusted to 0.1% (v/v).

T47D-ERβ cells were kindly provided by Dr. Jan-Åke Gustaffson, Karolinska Institute, Huddinge, Sweden. The T47D cell line is an epithelial, differentiated strain of the female ductal carcinoma cell line T47 and has a doubling time of approximately 32 hours.
By withdrawal of tetracycline, this stably transfected cell line expresses the estrogen receptor β together with EGFP (enhanced green fluorescent protein). Thus, the expression of the ERβ can be monitored upon UV illumination without any substrates or cofactors (Ström *et al*., 2004).
The cells are stored in liquid nitrogen and, prior to culture, they are thawed at 37°C, sterile-transferred to a Falcon tube containing warm complete DMEM (DMEM/F12 supplemented with 5% FBS, sodium bicarbonate, the previously indicated antibiotics and 1 μg/ml tetracycline) and then centrifuged for 5 minutes at 1'000 rpm. The cell pellet is resuspended in 10 ml warm complete medium, transferred to a 75 cm^2 culturing flask and put in the incubator at 37°C, saturated humidified air and 5% CO_2.
When cells have grown to subconfluence (80 – 95%), subculturing is carried out in a 1:3 ratio. First, the cells are washed with warm PUCK's EDTA. Then cells are trypsinised with 2 ml 1:30 Trypsin/PUCK's EDTA and the flask is gently

slapped with the palm of the hand to detach all cells. The cells are then collected in 10 ml of complete medium in a 50 ml Falcon tube and spun down for 5 minutes at 1'000 rpm. After removing the supernatant, the cell pellet is resuspended in 10 ml complete medium and the cells are split in a 1:3 ratio into 75 cm^2 culturing flasks. Before conducting an experiment, the cells are starved in estrogen-free and phenol red-free assay medium for 48 hours. For the ERβ assay the subcultered cells were divided into two groups: one group nourished with estrogen-free assay medium supplemented with 1 μg/ml tetracycline; the other group, for induction of ERβ, was grown in assay medium without tetracycline.

T47D.Luc cells (BioDetection Systems, Amsterdam, NL) were maintained in a 1:1 mixture of DMEM and Ham's F12 medium supplemented with sodium bicarbonate, 1 mM L-glutamine and 7.5% FBS. Before performing the ER-CALUX assay, T47D.Luc cells were seeded in 96 wells microtiter plates (Corning Inc, Garnd Island, USA) at a density of 5,000 cells per well in 100 μl phenol red-free medium containing 5% DCC-FBS. The outlining rows were left blank in order to prevent desiccation of cells.
After 48 hours in this estrogen-deprived milieu, with a medium renewal after 24 hours, the indicated test compounds, dissolved in DMSO, were added. Blank and solvent controls and a standard 17β-estradiol dose response were included on each plate. After 24 hours exposure time, the assay medium was sucked off and the cells were lysed and assayed for luciferase activity on a Dynex microplate luminometer (Legler *et al.* 1999).

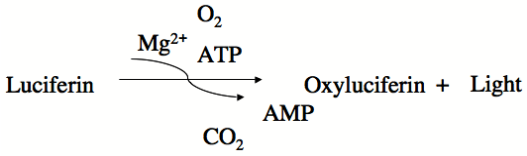
4.2 Cytotoxicity assay: resazurin cell viability assay (AlamarBlue® assay)

AlamarBlue (resazurin) is a safe (non-toxic, non-carcinogenic) aqueous dye to assess cell viability by monitoring the mitochondrial activity. Upon reduction by the mitochondrial coenzymes (FMNH2, FADH2, NADH, NADPH) the AlamarBlue® dye shows a change in colour and turns from blue (oxidised) into the red fluorescent resorufin derivative, thus indicating that the cells are vital.

The intensity of this colour change is measured in a microplate photometer at 565 nm for absorption and at wavelengths of 535 nm (excitation) and 595 nm (emission) for fluorescence. This assay was used to test the effect of the solvent DMSO as well as the cow- and soymilk extracts. Untreated cells served as negative control, cell-free wells served as blank controls. The assay was performed in 96-well-plates (Corning Inc., Grand Island, USA) with each well containing 5'000 cells and 100 μl of the specific assay medium. The outlining rows were left blank with complete growth medium.
After 24 hours of exposure, the test was performed by adding the AlamarBlue dye, 100 μl per well. The cells were further incubated (humidified air, 5% CO_2, 37 °C) until photometric measurements were carried out.

4.3 ER-CALUX Assay

The ER-CALUX (**c**hemical-**a**ctivated **luc**iferase gene e**x**pression) assay is a recombinant receptor reporter gene assay based on the induction of bioluminescence in the stably transfected cell line T47D.Luc (Legler *et al.*, 1999). It is a very sensitive, highly responsive and fast screening method for the estrogenicity of chemical compounds (detection limit for 17β-estradiol: 0.5 pM). The stably transfected cells display a chromosomally integrated pEREtata-Luc construct containing three tandem repeats of the consensus ERE sequence (5'-GGTCACTGTGACC-3') upstream of the TATA box. This promoter drives the expression of an enhanced luciferase reporter gene sequence derived from *Photinus pyralis*.
To perform the ER-CALUX assay, T47D.Luc cells were seeded with estrogen- and phenol red-free medium into 96 well plates at a density of 5'000 cells per well. The cells were exposed to the test compounds dissolved in DMSO resulting in a final DMSO concentration of 0.1%. After 24 hours, the medium was removed and the cells were lysed by addition of 100 μl of Lysis Buffer (Promega) and gentle shaking at room temperature for 10 minutes. Then the plates were assayed on a Dynex microplate luminometer for luciferase activity by automated addition of luciferin. Upon expression of the luciferase enzyme, luciferin is oxidised to oxyluciferin, thus generating photons that are detected in the luminometer. The reaction is as follows:



The emitted light has a wavelength of 562 nm and is directly proportional to the amount of luciferase produced, thereby reflecting the estrogenicity of the tested compounds. A 17β-estradiol standard curve (10 pM, 30 pM, 60 pM, 100 pM) as well as negative (cells with medium only) and blank controls (medium only) were included on all plates to calibrate the estrogenicity of the test compounds.

4.4 Isolation of total RNA

After 24 hours exposure to the compounds, cells are harvested by trypsinisation as described in section 3.1. After centrifugation, the cell pellet is transferred to ice and the total RNA is extracted according to the QIAGEN® RNeasy protocol. This extraction method involves the denaturation of cellular proteins with guanidine-thiocyanate, the precipitation of nucleic acids with ethanol and the selective binding of RNA to a silica-based membrane combined with high-speed microcentrifugation.
First, the cells and the membranes of their organelles are lysed with 600 μl of buffer RLT containing β-ME and guanidine-thiocyanate, which also inactivates RNase enzymes. The lysate is homgenised with QIAshredder spin columns that eliminate high molecular weight components like genomic DNA, only the eluate is collected and further processed. Subsequently, 600 μl ethanol are added to provide best binding conditions for RNA to the silica membrane in the RNeasy spin columns. The sample is transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 seconds at ≥ 10'000 rpm. The eluate is discarded and the membrane is subjected to three washing steps: once with 700 μl of buffer RW1

(containing guanidine-thiocyanate) and twice with 500 µl of buffer RPE (containing ethanol). Next, the spin column is placed into a new collection tube and centrifuged for 1 minute to dry the silica membrane and prevent carry-over of buffer into the total RNA eluate. The final elution is carried out into a new 1.5 ml collection tube with 30 µl RNase-free water, loaded directly onto the membrane, by centrifugation for 1 minute at 10'000 rpm. The purified total RNA is stored at –20 °C.

4.5 Quantification and quality control of total RNA

The total RNA concentration is determined with a UV-spectrophotometer and the total RNA quality (integrity and size distribution of the total RNA) is assessed with the Agilent 2100 Bioanalyzer.

UV-spectrophotometer:

Due to the fact that nucleic acids have their absorbance maximum at 260 nm, the concentration of the extracted total RNA can be determined by UV-spectrophotometry. The UV-spectrophotometer is calibrated wih 0.1 ml of Tris-HCl buffer. Each total RNA sample is diluted 1 : 200 with 10 mM Tris-HCl buffer, pH = 7.0 (0.5 µl sample RNA in 99.5 µl buffer) and absorbance is measured at 260/280 nm. An absorbance of 1 unit at 260 nm corresponds to 40 µg/ml of RNA. The RNA purity can be estimated by the ratio of the absorbance values at 260 nm and 280 nm (A_{260}/A_{280}), which should range from 1.8 to 2.1.

Agilent 2100 Bioanalyzer:

Total RNA samples consist by more than 80% of ribosomal RNA (rRNA) and only approximately 1-3% messanger RNA (mRNA). Therefore, a first quality assessment is based on the

28S : 18S ratio of rRNA. As the 28S and 18S rRNAs are approximately 5 kb and 2 kb in size, the theoretically possible ratio of 28S : 18S is 2.7 : 1, but a ratio of 2 : 1 is considered as a marker for intact, poorly degraded RNA. In practice, values higher than 1.7 suggest high quality RNA.

The Agilent 2100 bioanalyzer is an on-chip electrophoresis (microchannels graved in glass) system and uses a combination of micro-fluidics, capillary electrophoresis and fluorescent dyes that bind to nucleic acid to simultaneously evaluate RNA concentration and integrity. The RNA-associated fluorescence is monitored as RNA molecules move through a detector along the separation channel of the chip, the RNA and the fluorescence of these molecules is measured as they pass the detector. The resulting data is translated into gel-like images and electropherograms, which are used for the 28S : 18S ratio calculation (Figure 5).

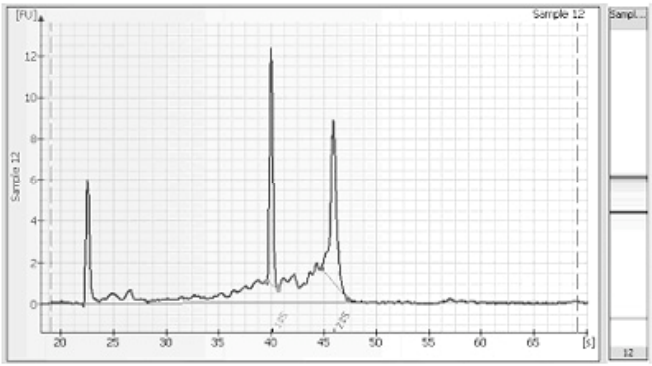


Figure 5: *high quality total RNA electropherogramme obtained in the Agilent 2100 bioanalyzer*

4.6 Complementary DNA synthesis

The GeneChip® One-Cycle cDNA Synthesis Kit and Poly–A RNA Control Kit are used for the conversion of mRNA to complementary DNA. The amount of total RNA used in each reaction is 5 μg. To have an endpoint control for the labelling process, exogenous positive controls in form of polyadenylated transcripts from *Bacillus subtilis* genes, which are absent in eukaryotic cells. These prokaryotic transcripts (lys, phe, thr, dap) are added to the RNA samples. Reaction mixtures containing total RNA (5 μg), 2 μl poly-A RNA control and 2 μl 50 μM T7-oligo(dT) primer (sequence: 5'–GGCCAGGCGG–$(dT)_{24}$–3'), were incubated in RNase-free water for 10 minutes at 70 °C, followed by cooling at 4 °C. Then the First-Strand Master Mix, consisting of 5x First-Strand Reaction Mix, 0.1 M DTT and 10 mM dNTPs, were added. After incubation at 42°C for 2 minutes, 1 μl SuperScript II (reverse transcriptase) was added and the tube was maintained at 42°C for 1 hour, followed by a cooling to 4 °C. Thereafter, 130 μl of the Second-Strand Master Mix (RNase-free water, 5x Second-Strand Reaction Mix, 10 mM dNTP, *E. coli* DNA ligase, *E. coli DNA* Polymerase I and RNase H) were added and the samples incubated for 2 hours at 16 °C. The reaction was supplemented with T4 DNA polymerase (2 μl) and incubated another 5 minutes at 16 °C. Then 10 μl 0.5 M EDTA are added and the samples were processed for complementary DNA purification.

4.7 Cleanup of complementary DNA

The GeneChip® Sample Cleanup kit is used to isolate the complementary DNA. For that purpose, 600 µl of cDNA Binding Buffer are added to the DNA preparation; 500 µl of the sample are applied onto a cDNA cleanup Spin Column, which is centrifuged at 10'000 rpm for 1 minute. The flow through is discarded, and the procedure repeated. The spin column is then transferred to a new collection tube and 750 µl of cDNA Wash Buffer are loaded onto the column, followed by 1 minute centrifugation at ≥ 10'000 rpm. To dry the silica membrane, the spin columns are centrifuged with opened caps for 5 minutes at maximum speed (20'000 rpm). For elution, the columns are transferred into 1.5 ml-collection tubes and 14 µl of cDNA Elution Buffer are loaded directly onto the spin column membrane. After a 1-minute incubation at room temperature, the column is centrifuged at 20'000 rpm for 1 minute to elute the complementary DNA.

4.8 Synthesis of biotin-labelled complementary RNA

The GeneChip® IVT Labeling Kit is used for complementary RNA synthesis. For that purpose, the cDNA eluate (~ 12 µl) is transferred into RNase-free microfuge tubes and the following components are added: RNase-free water to a final volume of 40 µl, 4 µl 10x IVT Labelling Buffer (containing spermidine), 12 µl IVT Labelling NTP Mix (including a synthetic biotin-conjugated nucleotide analog) and 4 µl IVT Labeling Enzyme Mix (containing RNA-polymerases). The reagents are carefully mixed and briefly centrifuged prior to incubation at 37 °C for 16 hours.

4.9 Cleanup and quantification of the biotinylated complementary RNA

The GeneChip® Sample Cleanup kit is used for purification of complementary RNA. Unincorporated NTPs are removed to guarantee an accurate determination of concentration and purity of the biotinylated cRNA. Sixty μl of RNase-free water are added to the complementary RNA and mixed by vortexing. Then, 350 μl IVT cRNA Binding Buffer are added, the sample is thoroughly mixed by vortexing. After adding 250 μl ethanol, the sample is applied to the IVT cRNA Cleanup spin column, which is centrifuged for 15 seconds at 10'000 rpm. The flow through is discarded and the spin column is transferred to a new 2-ml collection tube for washing with 500 μl of IVT cRNA Wash Buffer. Another 500 μl of ethanol (80%; v/v) are loaded and the spin column is centrifuged for 15 seconds at 10'000 rpm. The flow through is again discarded. Next, the spin column is centrifuged with open cap for 5 minutes at maximum speed (20'000 rpm) to allow for complete drying of the membrane. Finally, the spin column is transferred into a new collection tube and 11 μl of RNase-free water are loaded directly onto the membrane followed by a 1-minute centrifugation at maximum speed to elute the biotinylated complementary RNA. This procedure is repeated once.
The complementary RNA is quantificated by UV spectrophotometry and the over-all quality is assessed with the Agilent 2100 Bioanalyzer as described in 3.2. The total quantity of RNA is determined using the following equation.

$$\text{adjusted cRNA yield} = RNAm - (\text{total } RNAi) \times (y)$$

RNAm = amount of cRNA measured after IVT in μg
total RNAi = starting amount of total RNA in μg
y = fraction of cDNA used for the IVT reaction (12 μl)

4.10 Fragmentation of the complementary RNA

Fragmentation of complementary RNA is achieved by metal-induced hydrolytic breakdown to 35-200 base fragments). For that purpose, 20 μg of complementary RNA are mixed with 8 μl of 5x Fragmentation Buffer (from the GeneChip® Sample Cleanup kit and RNase-free water to a final volume of 40 μl. The fragmentation mixture is incubated for 35 minutes at 94 °C.

4.11 Hybridisation on the probe array

The GeneChip Hybridisation, Wash and Stain kit is used for this step, according to manufacturer's instruction. The following components are used for the hybridisation cocktail: 5 μl Control Oligonucleotide B2 (3 nM) (used as a grid alignment reference), 15 μl 20x Prokaryotic Hybridisation Controls (bioB, bioC, bioD, cre), 150 μl 2x Hybridisation Mix, 3 μl herring sperm DNA (10 mg/ml), 3 μl BSA (50 mg/ml), 30 μl DMSO, 15 μg of the fragmented and labelled complementary RNA. The volume is adjusted to 300 μl with RNase-free water and the mixtures is heated to 99°C for 5 minutes.
The probe arrays are wetted with 200 μl of 1x Hybridisation Buffer and incubated for 10 minutes at 45°C in the hybridisation oven shaking at 60 rpm. The Hybridisation mixture is incubated for 5 minutes at 45°C and clarified by centrifugation (20'000 rpm). Then the Hybridisation Buffer is removed from the probe arrays and replaced by 200 μl of the hybridisation mixture. The probe arrays are hybridised for 16 hours at 4 °C in the oven shaking at 60 rpm.

4.12 Washing, staining and data acquisition

After 16 hours of hybridisation, the cocktail is removed and the arrays are washed following the manufacturer's instructions in the Fluidics Station 400. For staining, the SAPE and antibody solutions are prepared as follows:

SAPE solution
600 μl 2x Stain Buffer, 48 μl 50 mg/ml BSA, 12 μl 1 mg/ml streptavidin phycoerythrin (SAPE) and 540 μl DEPC-treated H_2O are mixed and divided into two aliquots of 600 μl each. The SAPE solution has to be protected from light.

Antibody solution
300 μl 2x Stain Buffer, 24 μl 50 mg/ml BSA, 6 μl 10 mg/ml Goat IgG, 3.6 μl 0.5 mg/ml biotinylated antibody (anti-streptavidin) and H_2O to a final volume of 600 μl.

After inserting the probe arrays into their designated module of the Fluidics Station 400, the tubes are placed into the appropriate sample holders of each module (two times 600 µl SAPE solution and one 600 µl antibody solution per module). When washing and staining of the arrays is completed, the probe arrays are checked for large air bubbles that might constrain the scanning, the glass surfaces of the probe arrays are gently cleaned with a non-abrasive towel and, to prevent leakage of fluids (Wash Buffer A), the separations of the probe array cartridges are sealed with Tough Spots. Then the probe array cartridges are inserted in the scanning revolver of the GeneChip® Scanner 3000 and scanning of the arrays is performed at a pixel value of 3 µm and 570 nm wavelength. The scanner is controlled by GCOS (Gene Chip Operating Software).

4.13 Data analysis

Microarray quality assessment, condensing of the probe sets, data normalization and filtering were conducted using the Expressionist software (Genedata AG, Basel, Switzerland). T-tests were performed between controls and treated cells to assess the statistical significance of differentially expressed genes. False discovery rates were determined according to Benjamini-Hochberg (1995). Finally, the means of 3-5 replicates were imported into a Microsoft Excel file for graphical representation and determination of correlation coefficients. The "Gene Ontology" database (www.geneontology.org) was consulted for the molecular function of each transcript and only gene products with a known or inferred function are displayed in the figures.

4.14 Extraction and analysis of the milk samples

The sample preparation procedure developed for extracting phytoestrogens from milk, including the removal of endogenous estrogen hormones, has been adopted from Courant *et al*. (2007). Briefly, samples (cow milk or soymilk obtained from a local retailer) were extracted in 10 ml aliquots with acetate buffer (2 M, pH 5.2) and acetone. After centrifugation, the supernatants were collected and the acetone phase was evaporated under a nitrogen stream. Subsequently, an enzymatic hydrolysis was carried out by overnight incubation (52° C) with a purified *Helix pomatia* preparation (Sigma, St. Louis, MO, USA). This deconjugation step was followed by purification through two successive cartridges combining a reverse (C_{18}) and a normal (silica) stationary phase

(SDS, Peypin, France). After evaporation of the methanol eluate, the remaining residues were reconstituted in 30 μl of dimethyl sulfoxide (DMSO) for the cell culture experiments. Separate cow milk and soymilk samples were subjected to the same procedure, except that a deuterated compound (daidzein-d_3) was included as internal standard. These samples were analyzed by liquid chromatography-tandem mass spectrometry for the quantitative determination of phytoestrogens (Antignac *et al*. 2003). The 17β-estradiol measurements were performed according to a previously described method (Courant *et al*. 2007).

4.15 Real-time RT-PCR

PCR quantifications were carried out to validate the microarray hybridization results. Primers for the selected transcripts were obtained from Applied Biosystems. Briefly, 100 ng of complementary DNA were mixed with 100 nM of forward and reverse primers in a final volume of 25 μl. The reactions were performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) for 45 cycles (95 ºC for 15 sec, 60 ºC for 1 min) after an initial incubation time of 10 minutes at 95ºC. The fold change in the expression of each gene was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001), with the abundant glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript as an endogenous control.

5. Results

5.1 Sample preparation and analysis

After liquid extraction and enzymatic deconjugation, the soy and cow milk samples (10 ml each) were subjected to a two-step fractionation procedure using reverse phase (C_{18}) and normal phase (silica) cartridges. The quantitative analysis by liquid chromatography coupled to tandem mass spectrometry confirmed that soymilk contains large quantities of the isoflavones genistein and daidzein, whereas cow milk is characterized by the presence of low levels of enterolactone together with trace amounts of other phytoestrogens (Table 2). Additional measurements by gas chromatography-mass spectrometry verified that endogenous estrogen hormones, including 17β-estradiol, were removed from the cow milk sample during the final solid-phase extraction step. For the subsequence cell culture experiments, each isolate from a 10 ml sample was reconstituted in 30 μl dimethyl sulfoxide (DMSO), which proved to be compatible with the solubility properties of the various phytoestrogens.

	Concentration (ng/ ml)	
	Soy milk	Cow milk
Daidzein	1233	8.3
Genistein	5175	5.6
Equol	nd	2.6
Formononetin	9.6	1.8
Biochanin A	nd	nd
Glycitein	98.5	nd
Enterolactone	1.1	5.2
Matairesinol	nd	nd
Coumestrol	nd	nd
Resveratrol	nd	nd

Table 2:
Concentrations (ng/ml) determined for the target phytoestrogens in the analysed soymilk and cow milk samples (nd, not detected at the limit of detection, which varied from 0.05 to 0.7 ng/ ml depending on the compound)

5.2 Cell viability assay, ER-CALUX

For the cell viability assay, human MCF-7/BUS and T47D breast cancer cells were initially exposed to increasing concentrations of the soy and cow milk extracts dissolved in DMSO (0.05%, 0.1% and 0.3% (v/v)) or the same amounts of DMSO alone. Cell viability was tested 24 hours later with the AlamarBlue® TM cytotoxicity assay by measuring the intracellular mitochondrial activity, which is used as an indicator of metabolic activity. The resulting dose responses demonstrated that no cytotoxic reactions were triggered when the cell culture medium contained up to 0.3% of milk extract dissolved in DMSO.

Next, the soy and cow milk extracts were tested for their overall estrogenic activity using a standard reporter gene assay, i.e., the ER-CALUX assay. For that purpose, we exploited a stably transfected carcinoma cell line (T47D.Luc) that carries a chromosomally integrated reporter gene sequence (Legler *et al.,* 1999). This artificial construct drives the expression of firefly luciferase in response to ER activation and ER binding to the ERE. Thus, to monitor estrogenic activity, cell lysates were examined for luciferase activity after a 24-h treatment with progressively increasing concentrations of 17β-estradiol and the different extracts added to the cell culture medium. In all treatments, the final concentration of the DMSO solvent was set to 0.1%.

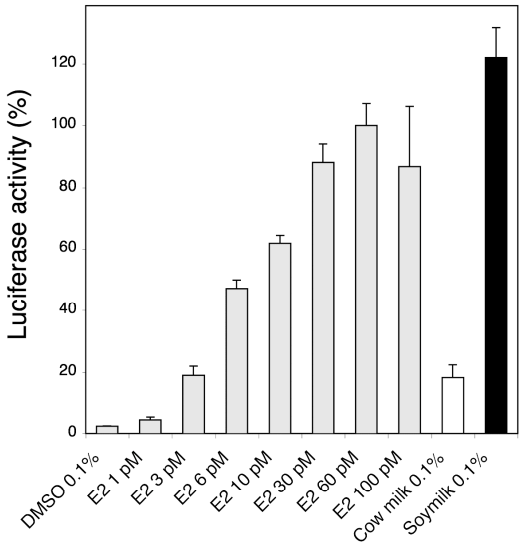


Figure 6:
Luciferase reporter gene assay (ER-CALUX)
T47D.Luc cells were incubated with 17β-estradiol (E2, at concentrations of 1-100 pM), as well as soy or cow milk extracts. The final concentration of the DMSO solvent was 0.1%. ER activation was determined by measuring the luciferase induction from a minimal promoter containing repeats of EREs (mean values of 5-6 independent experiments). The results are shown in percentages of the induction observed with 60 pM 17β-estradiol.

In a series of control reactions, the synthetic promoter mediated a dose-dependent luciferase induction in response to the 17β-estradiol standard. This estrogenic effect reached peak levels at a hormone concentration of 60 pM (Fig. 6). A similar level of reporter gene induction was observed in the cells incubated

with soymilk extract. The corresponding cow milk extract resulted in a considerably lower reporter gene induction compared to the soymilk extract, which is, however, in agreement with its marginal phytoestrogen content (Fig. 6). These responses to the treatment with 17β-estradiol or soymilk extract were completely suppressed by the addition of the ER antagonist ICI 182,780 at a concentration of 0.1 μM.

5.3 Expression profiles induced by soy and cow milk samples

The MCF7/BUS breast cancer cell line is markedly more responsive to estrogenic stimuli than T47D cells, thus delivering a wider range of estrogen-regulated genes as well as larger amplitudes of expression changes (Buterin *et al.*, 2006). Therefore, MCF7/BUS cells were used to perform genome-wide analyses of endogenous transcripts after a treatment with phytoestrogen mixtures reconstituted in culture medium. The target cells were incubated in triplicates with soymilk or cow milk extracts to reach a final solvent concentration of 0.1% (v/v). After 24-h exposure times, a fraction of RNA from each sample was analysed using Affymetrix microarrays that display the sequences of 47'400 human transcripts. To identify genes that are susceptible to ER regulation, these microarray data were normalized and subjected to statistical analysis. Also, in view of the large number of regulated genes, the expression data were filtered for transcripts that exhibited at least a fivefold change relative to the solvent control, thereby eliminating the vast majority of gene products that are more moderately affected or not altered at all following the phytoestrogen treatment. The significance threshold was $P <$

0.01, yielding false discovery rates in the range of 0.02 - 0.1 (Benjamini & Hochberg, 1995).

The incubation with the soymilk extract resulted in a total of 358 different transcripts that were at least fivefold up- or down-regulated compared to the solvent control group. In contrast, the gene expression profile induced by the cow milk sample deviated only marginally from the background transcriptional pattern observed in the solvent control group. In this case, only six transcripts were affected by more than fivefold changes, thus reflecting the much lower phytoestrogen concentration and content in cow milk.

The transcripts displaying the highest amplitude of regulation in response to the treatment with soymilk phytoestrogens are shown in Figure 7. The majority of these transcripts encode for proteins involved in DNA metabolism (RRM2, TYMS, TK1; see legend to Fig. 7 for abbreviations), DNA replication or recombination (MCM10, CDT1, TOP2A, PRIM1, RAD51), cell division cycle (CCNA2, CDC2, CDC6, TTK), chromosome segregation and centromere function (AURKB, KNSL7, Spc24, Spc25, CDCA1, KNTC2, KIF2C, ESPL1, ASPM) or inhibition of apoptosis (BIRC5). Together with the overexpressed proto-oncogenes MYBL1 and MYBL2 as well as two different proliferation markers (MKI67 and OIP5), this transcriptional profile reflects the typical mitotic signature observed in estrogen-stimulated breast cancer cells (Lobenhofer *et al.*, 2002; Coser *et al.*, 2003; Frasor *et al.*, 2003; Vendrell *et al.*, 2004; Buterin *et al.*, 2006; Lavigne *et al.*, 2007).

When the expression changes resulting from the soymilk extract exposure where juxtaposed to the expression changes induced by the 17β-estradiol treatment, their striking similarity became apparent. In fact, all transcripts that were increased by incubation with the soymilk extract were also up-regulated

following the treatment with 17β-estradiol (Fig. 8). To analyse this presumed relationship in more quantitative terms, the messenger RNA profile induced by the soymilk extract was plotted against the corresponding values obtained with 17β-estradiol.

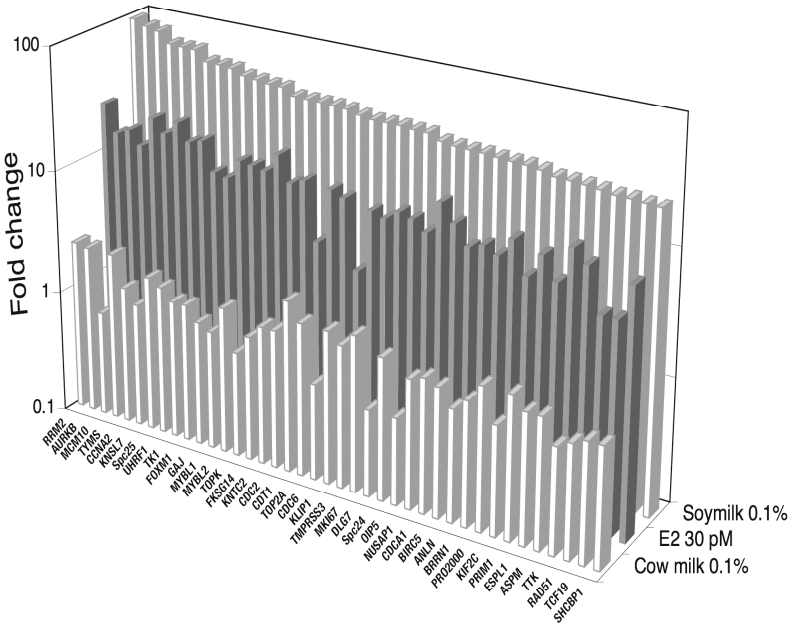


Figure 7:
Comparison of genome-wide transcriptional changes in MCF-7/BUS cells exposed to soy and cow milk extracts or 17β-estradiol. Transcripts with the highest amplitude of induction following a treatment with 0.1 % soymilk extract in comparison to the level of the same transcripts following the treatment with cow milk and 30 pM 17β-estradiol.

Abbreviations in Figure 7:
RRM2, *ribonucleotide reductase M2*; AURKB, *aurora kinase B*; MCM10, *minichromosome maintenance-deficient 10*; TYMS, *thymidylate synthetase*; CCNA2, *cyclin A2*; KNSL7, *kinesin-like 7*; Spc25, *kinetochore protein Spc25*; UHRF1, *ubiquitin-like, containing PHD and RING finger domains 1*; TK1, *thymidine kinase 1*; FOXM1, *forkhead box 1*; GAJ, *GAJ protein;* MYBL1, *myeloblastosis oncogene-like 1*; MYBL2, *myeloblastosis oncogene-like 2*; TOPK, *T-LAK cell-originated protein kinase*; FKSG14, *leucine zipper protein FKSG14*; KNTC2, *kinetochore associated 2*; CDC2, *cell division cycle 2*; CDT1, *DNA replication factor*; TOP2A, *topoisomerase II-α*; CDC6, *cell division cycle 6*; KLIP1, *KSHV latent nuclear antigen interacting protein 1*; TMPRSS3, *transmembrane protease serine 3*; MKI67, *antigen identified by monoclonal antibody Ki-67*; DLG7, *discs large homolog 7*; Spc24, *kinetochore protein Spc24*; OIP5, *Opa-interacting protein 5*; NUSAP1, *nucleolar and spindle-associated protein 1*; CDCA1, *cell division cycle-associated 1*; BIRC5, *baculoviral IAP repeat-containing 5*; ANLN, *anilin*; BRRN1, *barren homolog*; PRO2000, *PRO2000 protein*; KIF2C, *kinesin family member 2C*; PRIM1, *primase 1*; ESPL1, *extra spindle poles-like 1*; ASPM, *abnormal spindle-like microcephaly*; TTK, *TTK protein kinase*; RAD51, *RAD51 homolog*; TCF19, *transcription factor 19*; SHCBP1, *ortholog of mouse Shc SH2-domain binding protein 1.*

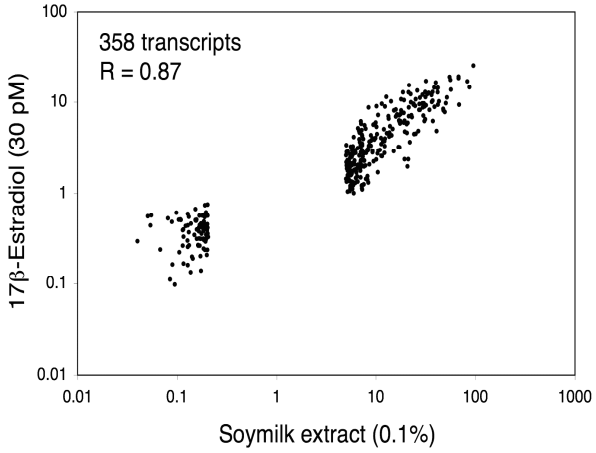


Figure 8:
Linear regression analysis of pairs of data obtained with 0.1 % soymilk extract and 30 pM 17β-estradiol. A fivefold change (up- or down-regulation) in the soymilk-treated samples relative to the controls was used as the cut-off to filter the data. A total of 358 transcripts passed the statistical threshold of $P < 0.01$.

All transcripts that showed at least a fivefold up- or down-regulation in the phytoestrogen treatment group were included in this analysis. The general threshold for statistical significance was $P < 0.01$. Figure 8 shows that the data points in this comparison grouped in two distinct clusters reflecting those genes that were over-expressed and those that were under-expressed relative to the solvent control group. A linear regression analysis of all 358 pairs of data yielded an overall correlation coefficient of $R = 0.87$, thereby exceeding the values ($R \approx 0.6$) found in another similar study that compared expression changes induced by soy extracts with those resulting from 17β-estradiol exposure (Ise *et al.*, 2005). The close correspondence of expression values, demonstrated in

Figure 8, is supporting the notion that soy phytoestrogens and 17β-estradiol induce nearly identical transcriptional responses in MCF7/BUS cells. Thus, in contrast to previous reports (Wang *et al.*, 2004; Ise *et al.*, 2005), we found that the transcriptional machinery of this breast cancer cell line responds in a very similar manner to different estrogenic stimuli.

5.4 Real-time PCR: Validation of microarray results

Real-time reverse transcriptase-PCR (RT-PCR) assays were carried out on representative sequences to confirm the tight correlation between the expression profiles induced by soy phytoestrogens and 17β-estradiol. The following transcripts were tested subsequent to treatment with either soymilk phytoestrogens or the physiologic hormone 17β-estradiol: RRM2 (ribonucleotide reductase M2 polypeptide), CDC2 (cell division cycle 2), TTK (a protein kinase) and UAGT5 (UDP-N-acetylglucosaminyltransferase 5).
The exposure to soy phytoestrogens was performed with three different proportions of extract in the cell culture medium, between 0.001% and 0.1% (v/v). In view of the phytoestrogen contents listed in Table 2, these soymilk residues translate to genistein concentrations ranging from 0.1 to 10 μM. Incubations with the 17β-estradiol reference were performed using the standard concentration of 30 pM. After normalization with the constitutive GAPDH (glyceraldehyde-3-phosphate dehydrogenase) transcript, expression values were transformed as the ratio of messenger levels between treated cells and solvent controls. A linear regression analysis of the resulting RT-PCR values yielded higher correlation coefficients of R =

0.92 – 0.98, thus confirming a tight correlation between the transcriptional changes induced by soy phytoestrogens and the physiologic estradiol hormone (Figure 9).

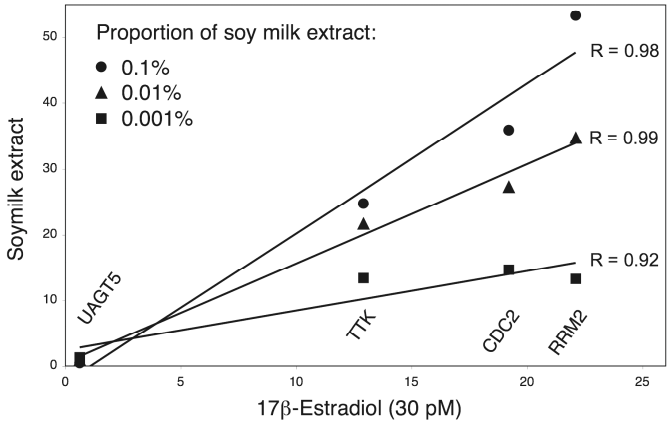


Figure 9:
RT-PCR analysis of selected transcripts that were regulated following exposure of MCF7/BUS cells to estrogenic stimuli. The results obtained with three different concentrations of the soymilk extract (0.001%, 0.01%, 0.1%; v/v) were plotted against the corresponding values obtained in the experiment with 17β-estradiol (30 pM). The fold changes were measured using the GAPDH transcript as an endogenous control (mean values of 4 – 5 independent determinations). The linear regression analysis to determine the degree of similarity between the transcriptional changes induced by soymilk extract and 17β-estradiol yielded correlation coefficients of R = 0.92 – 0.98.

5.5 Expression profiles induced by single phytoestrogens

Buterin *et al.*, 2006 demonstrated that genistein, the predominant soy phytoestrogen, induces global gene expression profiles in both MCF7/BUS and T47D cells that are indistinguishable from the transcriptional changes resulting from 17β-estradiol treatments. A similar convergence was now obtained when we assessed the response of MCF7/BUS cells to daidzein, another major soy phytoestrogen, tested at a concentration of 1 μM (Fig. 10-1). Subsequently, this study was extended to different categories of phytoestrogens including coumestrol, resveratrol and enterolactone. The specific criteria for inclusion of the transcripts into the correlation analyses of Figure 10 were again a fold change greater than 5 and a corresponding *P* value less than 0.01. Compared to the daidzein treatment, the number of significantly regulated transcripts was slightly higher in the coumestrol experiment (Figure 10-2) and markedly reduced in the resveratrol and enterolactone treatments (Figures 10-3 and 10-4). However, a direct comparison with the effects of 17β-estradiol on the same human genes yielded correlation coefficients of $R = 0.85 – 0.92$, further supporting the idea that, at least in a low-dose range, all kinds of phytoestrogens generate expression profiles in human breast cancer cells that are identical to the transcriptional pattern elicited by a 17β-estradiol stimulus.

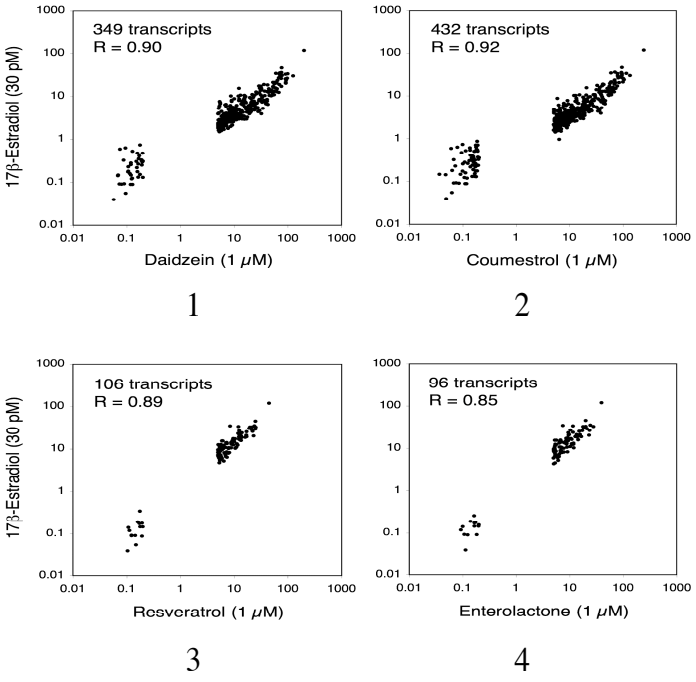


Figure 10:

Comparison between 17β-estradiol and single phytoestrogens treatment.

MCF7/BUS cells were incubated with 1 µM of the indicated phytochemicals (3 - 4 independent replicates). The resulting expression changes were plotted against the corresponding data obtained with 17β-estradiol (30 pM). A fivefold change in the phytoestrogen-treated samples was used as the cut-off to filter the data, and the number of significantly ($P < 0.01$) regulated transcripts is indicated for each compound. The quantitative relationship between the different molecular fingerprints was determined by linear regression analyses, yielding correlation coefficients of $R = 0.85 - 0.92$

5.6 Influence of ERβ on expression profiles

RT-PCR quantifications with oligonucleotide primers specific for each ER subtype showed that the predominant mRNA in MCF7/BUS and T47D cells is the one coding for ERα, whereas ERβ transcripts remained undetectable (data not shown). These findings are consistent with previous studies reporting the presence of only trace amounts of ERβ transcripts in T47D cells and essentially no such transcripts in MCF7/BUS cells (Legler *et al.*, 1999; Lobenhofer *et al.*, 2002). By immunoblotting methods it has also been shown that there is no detectable ERβ protein in T47D cells (Ström *et al.*, 2004). The lack of an appropriate breast cancer cell line containing significant amounts of ERβ protein can be circumvented by taking advantage of a genetically modified cell line (T47D-ERβ) in which a tetracycline-regulated construct drives the expression of a full-length human ERβ sequence (Ström *et al.*, 2004; Matthews, 2006). We used this stably transfected T47D-ERβ cell line to examine the contribution of ERβ to the transcriptional reprogramming triggered by phytoestrogens.

T47D-ERβ cells were exposed to 1 μM coumestrol because this particular phytochemical displays the highest affinity for ERβ among all phytoestrogens tested to date (Kuiper et al. 1997; Kuiper et al. 1998; Bovee *et al.*, 2004; Mueller *et al.*, 2004). The transcriptional variances following this coumestrol treatment were scrutinised in the presence of tetracycline, i.e. under conditions that suppress the expression of ERβ, as well as after tetracycline withdrawal, resulting in promoter activation and ERβ co-expression. In the absence of tetracycline, the level of messenger RNA coding for ERβ is 4 – 5 times higher than that of the corresponding ERα transcripts (Ström *et al.*, 2004), hence confirming that this inducible

system leads to expression of both receptor subtypes in substantial quantities. To eliminate possible confounding effects due to the antibiotic used for ERβ suppression, the fingerprints obtained in the presence of tetracycline were compared to appropriate solvent controls containing the same concentration of antibiotic. Conversely, the fingerprints obtained after tetracycline withdrawal, consequently leading to ERβ expression, were determined against corresponding controls without tetracycline in the medium.

The distinctly different gene expression profiles induced by coumestrol in the absence or in the presence of ERβ are illustrated in Figure 11. To simplify the representation of data, the graph of Figure 11 shows only those transcripts that were up-regulated by a fold change greater than 10 in at least one of the treatment groups ($P < 0.01$ for all transcripts). The response obtained in T47D-ERβ cells containing ERα alone involves for example an overexpression of IL20 (see legend to Figure 11 for abbreviations), CXCL2, IGFBP4, MYB or TFF1 (also known as pS2). The range of regulated target genes did not change when, in the absence of tetracycline, both ERα and ERβ were expressed concomitantly in the same cell line. However, the induction of many transcripts was attenuated in the presence of ERβ, indicating that this additional receptor subtype is able to partially suppress the transactivation functions exerted by ERα. Conversely, in the presence of ERβ, other transcripts were regulated with larger amplitudes of induction than in the cells expressing ERα only. This second category of genes, which become more responsive in the presence of ERβ, include for example tripin, TOP2A (see legend to Figure 10 for abbreviations), the cyclin-dependent kinase inhibitors CDKN3 and CDKN2C as well as multiple members of the kinesin family (KNSL7, KIF20A, KIF14).

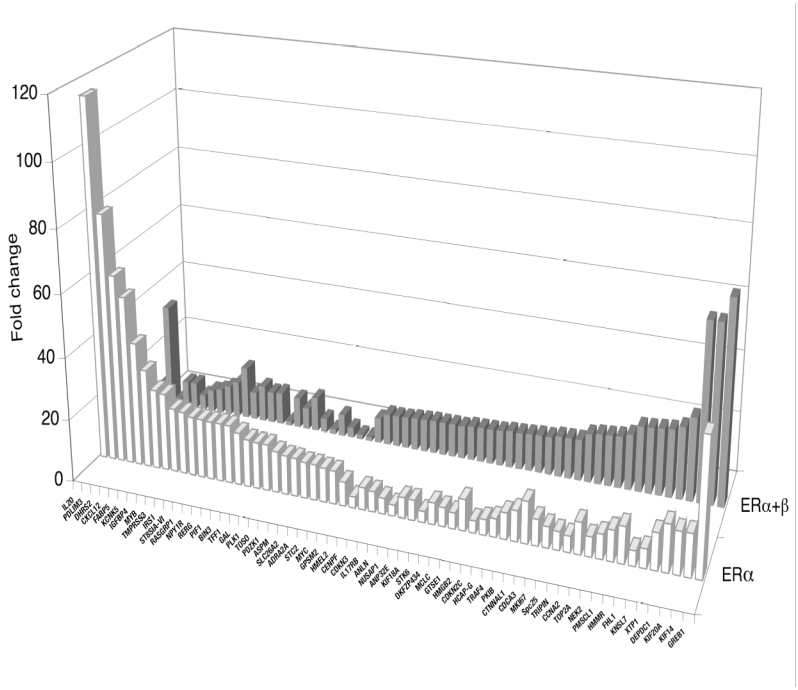


Figure 11:
Impact of ERβ on gene expression profiles. T47D-ERβ cells were exposed to 1 µM coumestrol either in the presence or in the absence of tetracycline. The fold changes of each transcript (mean values of three independent experiments) were calculated using, as the reference, solvent controls with or without tetracycline. Light grey: gene expression profile in cells containing only ERα (in the presence of tetracycline). Dark grey: gene expression profile induced in cells containing both ERα and ERβ (in the absence of tetracycline).

Abbreviations in Figure 11:
IL20, *interleukin 20*; PDLIM3, PDZ and LIM domain 3; DHRS2, *dehydrogenase/reductase (SDR family) member 2*; CXCL12, *chemokine ligand 12*; FABP5, *fatty acid binding protein 5*; KCNK5, *potassium channel, subfamily K, member 5*; IGFBP4, *insulin-like growth factor binding protein 4*; MYB, *myeloblastosis oncogene*; TMPRSS3, *transmembrane protease serine 3*; IRS1, *insulin receptor substrate 1*; ST8SIA-VI, *alpha 2,8-sialyltransferase*; RASGRP1, *RAS guanyl releasing protein 1*; NPY1R, *neuropeptide Y receptor 1*; RERG, *RAS-like estrogen-regulated growth inhibitor*; PIF1, *DNA helicase homolog 1*; BIN3, *bridging integrator 3*; TFF1, *pS2, trefoil factor 1*; GAL, *galanin*; PLK1, *polo-like kinase 1*; TOSO, *regulator of Fas-induced apoptosis*; PDZK1, *PDZ domain containing 1*; ASPM, *abnormal spindle-like microcephaly*; SLC26A2, *solute carrier family 26 member 2*; ADRA2A, *adrenergic alpha-2A receptor*; STC2, *stanniocalcin 2*; MYC, *myelocytomatosis oncogene*; GPSM2, *G-protein signaling modulator 2*; CENPF, *centromere protein F*; CDKN3, *cycline-dependent kinase inhibitor 3*; IL17RB, *interleukin 17 receptor B*; ANLN, *anilin*; NUSAP1, *nucleolar and spindle-associated protein 1*; ANP32E, *acidic nuclear phosphoprotein 32 family E*; RACBAP1, *Rac GTPase activating protein 1*; STK6, *serine-threonine kinase 6*; KIF18A, *kinesin family member 18A*; MCLC, *Mid 1-related chloride channel 1*; GTSE1, *G2 and S phase-expressed 1*; HMGB2, *high-mobility group box 2*; CDKN2C, *cyclin-dependent kinase inhibitor 2C*; HCAP-G, *chromosome condensation protein G*; TRAF4, *TRAF4-associated factor* 1; PKIB, *protein kinase inhibitor beta*; CTNNAL1, *catenin alpha-like 1*; CDCA3, *cell division cycle-associated 3*; MKI67, *antigen identified by monoclonal antibody Ki-67*; Spc25, *kinetochore protein Spc25*; CCNA2, *cyclin A2*; TOP2A, *topoisomerase II-*α; NEK2, *never in*

mitosis gene a-related kinase 2; PMSCL1, *polymiositis-scleroderma autoantigen 1*; HMMR, *hyaluron-mediated motility receptor*; FHL1, *four and a half LIM domains 1*; KNSL7, *kinesin-like 2*; XTP1, *HBxAG transactivated protein 1*; DEPDC1, *DEP domain containing 1*; KIF20A, *kinesin family member 20A*; KIF14, *kinesin family member 14*; GREB1, *GREB1 protein.*

As many phytoestrogens display a selective binding affinity for ERβ whereas 17β-estradiol has approximately the same affinity for both major estrogen receptor subtypes, ERα and ERβ, respectively (Kuiper *et al.,* 1997; Kuiper *et al.,* 1998), we next explored the hypothesis that the preferential interaction of these phytochemicals with ERβ may mediate a more distinctive transactivation function. These experiments were again carried out with coumestrol as this compound displays the highest affinity for ERβ among all tested phytoestrogens (Bovee *et al.,* 2004, Mueller *et al.*, 2006). The resulting expression changes were filtered for transcripts that exhibit at least a threefold induction relative to the untreated controls. A statistical threshold of *P* less than 0.05 was applied to the differentially expressed transcripts. In T47D-ERβ cells expressing only ERα, in the presence of tetracycline, we observed the already known tight correlation between the expression profiles generated by 17β-estradiol and coumestrol, with 79 transcripts matching the filtering criteria (Figure 12-1). In fact, upon linear regression analysis, the correlation coefficient between the 17β-estradiol and coumestrol data reached a value of $R = 0.90$. For the same transcripts, this correlation coefficient was reduced to $R = 0.70$ when the cells, in the absence of tetracycline, were expressing the ERβ subtype (Figure 12-2). Also, the slope of the linear regression decreased from 0.7 (Figure 12-1) to 0.3 (Figure 12-2), reflecting a diminished response to 17β-estradiol, relative to

the effects of coumestrol, in cells expressing the ERβ subtype. Accordingly, the similarity of transcriptomic patterns generated by 17β-estradiol and coumestrol is reduced in the presence of ERβ, suggesting that this particular receptor subtype may indeed mediate differential cellular reactions upon stimulation by phytoestrogens.

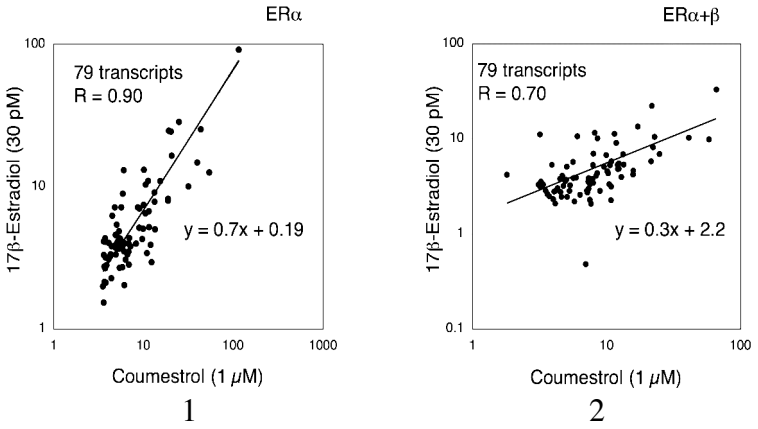


Figure 12:
Diverging expression profiles in the presence of ERβ. T47D-ERβ cells were challenged with 17β-estradiol (30 pM) or coumestrol (1 μM), either in the presence or in the absence of tetracycline. The fold changes of each transcript (mean values of three independent experiments) have been calculated using, as the reference, solvent controls with or without tetracycline. (1) Comparison of gene expression profile in cells containing only ERα (in the presence of tetracycline). (2) Comparison of expression profiles in cells containing both ERα and ERβ (in the absence of tetracycline). These graphs illustrate that ERβ decreases both the correlation coefficient and the slope of the linear regression.

6. Discussion

Breast cancer has become the most common malignancy among American and European women (Rice & Whitehead, 2006). However, in Eastern countries such as Japan the incidence of breast cancer is only about one-third of that of Western populations. This difference has often been attributed to a much higher dietary intake of soy phytoestrogens (Konstantakopoulos *et al.*, 2006; Martinez *et al.*, 2006; McCarty, 2006; Messina *et al.*, 2006). Unfortunately, despite the large amount of research conducted in the last years, no clear consensus has emerged regarding the preventive action of phytoestrogens against cancer. There is still no conclusive evidence that the ingestion of phytoestrogens is directly related to a reduced incidence of breast cancer, or whether phytoestrogens rather represent a biomarker of generally healthy diets (Martinez *et al.*, 2006; Rice & Whitehead 2006).
At low physiologic serum concentrations that are normally achieved by nutritional intake, phytoestrogens are likely to act through modulation of estrogen signaling. In fact, these observed serum concentrations (in the nM range) appear insufficient to inhibit tyrosine kinases or other enzymes that may provide alternative targets of phytoestrogen effects (McCarty, 2006). This low-dose estrogenic response is mediated by two members of the nuclear steroid receptor superfamily, ERα and ERβ. Both receptors constitute ligand-stimulated transcription factors that associate with co-regulatory partners to remodel chromatin and recruit the general transcription machinery to downstream genes (Katzenellenbogen & Katzenellenbogen, 2000; Hall *et al.*, 2001; Moggs and Orphanides, 2001; Safe, 2001). Although both receptors bind to the same consensus estrogen responsive element within gene promoters, ERα and ERβ have been

shown to exert partially antagonistic effects (Omoto *et al.*, 2003; Ström *et al.*, 2004).

Several findings converge on the idea that the proliferative stimulus on estrogen-dependent tissues, mediated by activation of ERα, can be opposed by the expression of ERβ. First, transgenic mice lacking ERβ are more susceptible than wild-type controls to develop markers of epithelial hyperplasia in the mammary gland (Förster *et al.*, 2002). Second, it has been observed that the mRNA coding for ERα is up-regulated during cancer progression, whereas the ERβ transcript is reduced in part via promoter methylation (Iwao *et al.*, 2000; *et al.*, 2000; Rody *et al.*, 2005; Park *et al.*, 2006). Third, the continued expression of ERβ in breast tumors is associated with low aggressiveness and improved survival rates compared to ERβ-negative counterparts (Hopp *et al.*, 2004). Fourth, activation of ERα promotes the growth of breast cancer cells both in culture and in animal models (Soto *et al.*, 1995; Hsieh *et al.*, 1998; Allred *et al.*, 2001; Ju *et al.*, 2006) but, when ERβ is restored using an appropriate expression vector, it exerts a negative effect on cell proliferation or even induces apoptosis (Omoto *et al.*, 2003; Skliris *et al.*, 2003; Ström *et al.*, 2004). This anti-proliferative action of ERβ correlates with the down-regulation of several factors involved in DNA replication and the cell cycle machinery (Lin *et al.*, 2007).

Contrary to 17β-estradiol, which does not discriminate between ERα and ERβ, phytoestrogens bind to ERβ with up to five times higher affinities compared to ERα (Kuiper *et al.*, 1997; Kuiper *et al.*, 1998). This finding appears relevant in view of the possible action of ERβ as a tumor suppressor because phytoestrogens may be able to trigger beneficial responses through their preferential interaction with the ERβ subtype. In contrast, such a protective effect is abrogated in cells that specialize on the expression of ERα with minimal residual

amounts of ERβ (Park *et al.*, 2006). This possible mechanism was supported when, in the present work, we analyzed the transcriptional fingerprints induced by soymilk extracts in human breast cancer cells. This type of soy product has been tested in the context of our study because of its high content in isoflavones with estrogenic activity, such that the findings may be extrapolated to a wide range of other soy-based foods and supplements. Unlike previous reports (Wang *et al.*, 2004; Ise *et al.*, 2005), we observed that such a natural mixture of soy phytoestrogens, as well as other types of phytoestrogens, induce a stereotyped expression fingerprint in breast cancer cells containing high levels of ERα but essentially no ERβ. Thus, in the absence of ERβ, all tested phytoestrogens result in essentially the same expression changes as those induced by the endogenous 17β-estradiol, and this recurrent genomic profile reflects the proliferative response mediated through ERα activation. This particular signature includes the up-regulation of many factors involved in cell cycle, DNA replication, chromosome segregation and inhibition of apoptosis. However, when the expression of ERβ is reconstituted using an inducible genetic system, the same breast cancer cells react in a different manner to phytoestrogen stimulation. First, the induction of many growth-promoting transcripts involved in the cell division cycle is attenuated compared to the genuine 17β-estradiol response and, second, there is a stronger induction of factors that arrest cell proliferation, such as for example inhibitors of cyclin-dependent kinases, thus further contributing to the inhibition of cell proliferation.

This mitigation of ERα-induced expression fingerprints may account for the presumed chemopreventive activity of phytoestrogens since, as indicated before, many of these compounds display particularly strong affinities for ERβ. In

view of our findings, we propose a biphasic activity of phytoestrogens during cancer development in estrogen-sensitive tissues. Accordingly, the presumed beneficial effects of phytoestrogens depend on the timing of exposure. Normally, phytoestrogens are able to slow down cell growth by activating ERβ, thereby generating an anti-proliferative expression signature. Due to the genetic instability of malignant tumor cells, however, the expression of ERβ may be abrogated by gene deletion or promoter methylation. In such late-stage cancer cells, phytoestrogens, in conjunction with other estrogenic chemicals, induce a transcriptional profile that promotes the proliferation of those clones that exhibit high amounts of ERα but little ERβ. Thus, in the early stage of carcinogenesis (with intact ERβ expression) phytoestrogen intake is more likely to exert beneficiary effects than in the later, ERα-dominated phases (Park *et al.*, 2006). As binding of phytoestrogens to the ERβ results in an increased transcription of genes related to cell-cycle arrest, the proliferative effects exerted through ERα could possibly be opposed by ERβ and tumor growth might come to cessation. In light of these considerations, the potentially beneficial effect of phytoestrogens should be re-evaluated, particularly in relation to risk groups (for example postmenopausal women) who are susceptible to the development of ERα-positive but ERβ-negative tumors arising from steroid hormone-dependent tissues (Cassidy, 2003).

Beyond the scope of this study, attentiveness should also be paid to another vulnerable risk group: newborns and infants. Although in most European countries the feeding of soy formula milk is restricted to infants with intolerance to regular formula, in the USA up to 36% of all formula-fed infants receive soymilk (Chen *et al.*, 2004; Tan *et al.*, 2006; Bernbaum *et al.*, 2007; Cao *et al.*, 2009). These children display plasma

levels of isoflavones that are up to 200-fold higher than those of infants fed cow milk formula or human breast milk. The increased isoflavone levels were found to be higher than those that are thought to exert physiological effects in adults (Chen *et al*., 2004). Critical targets for adverse isoflavone effects in newborns and infants are ER-expressing cells in the gastrointestinal tract and in the central nervous system (Burton *et al*., 2002; Yellayi *et al*., 2002; Patisaul, 2005; Tan. *et al*., 2006; Bernbaum *et al*., 2008; Antignac *et al*., 2008).

7. Abbreviations

β-ME	=	β-Mercaptoethanol
cDNA	=	Complementary DNA
Coum	=	Coumestrol
Daid	=	Daidzein
DBD	=	DNA-binding domain
DMEM	=	Dulbecco´s modified Eagle medium
DMSO	=	Dimethylsulfoxide
DNA	=	Desoxyribonucleic acid
dNTPs	=	Deoxyribonucleotide triphosphates
ds	=	Double-stranded
DTT	=	Dithiothreitol
E_2	=	17β-Estradiol
Ent	=	Enterolactone
ERα	=	Estrogen Receptor α
ERβ	=	Estrogen Receptor β
ERE	=	Estrogen Response Element
FBS	=	fetal bovine serum
H_2O_2	=	hydrogen peroxide
ICI 182,780	=	Fulvestrant, steroidal antiestrogen
IVT	=	in vitro transcription
MCF-7/BUS	=	female mamma carcinome cell line, strain 7, subtype BUS,
mRNA	=	messenger RNA
PBS	=	Phosphate-buffered saline
Res	=	Resveratrol
RNA	=	Ribonucleic acid
rpm	=	Revolutions per minute
ss	=	Single-stranded
T47D	=	Female ductal carcinoma cell line,

		differentiated epithelial substrain
T47D.Luc	=	Cell line T47D, stably transfected with an estrogen-responsive luciferase reporter gene
Tet	=	Tetracycline
Tris-EDTA	=	Tris-ethylenediamine tetraacetic acid solution
tRNA	=	total RNA, also: transfer RNA

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